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Synthesis and Application of a 5′-Aldehyde Phosphoramidite for Covalent Attachment of DNA to Biomolecules

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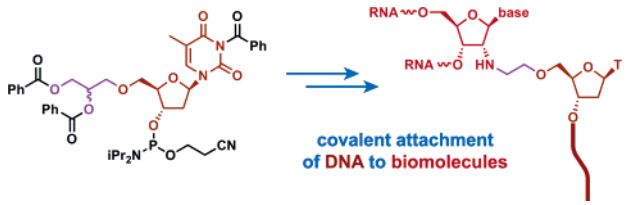

We recently reported the use of covalently attached DNA as a structural constraint for rational control of macromolecular conformation. Reductive amination was employed to attach each strand of the duplex DNA constraint to RNA, utilizing an aldehyde tethered to the 5′-terminus of the DNA. Here we describe the synthesis of a thymidine phosphoramidite that has the 5′-tethered aldehyde masked as a 1,2-diol. We also describe optimized reductive amination conditions for linking 5′-aldehyde-DNA with 2′-amino-2′-deoxy-RNA. These procedures should be generally applicable for attaching DNA to biomolecules.

The DNA constraint strategy as applied to control RNA folding requires covalent attachment of DNA oligonucleotides to an RNA macromolecule.[1,2] To enable site-specific attachment of DNA, the coupling reaction must use functional groups that are compatible with those found naturally in DNA and RNA. Several linkage reactions have been explored for attaching DNA oligonucleotides to small organic molecules and large biomolecules, such as reductive amination and formation of oximes, hydrazones, and disulfides.[3−5] Among these approaches, reductive amination via the formation of an imine (Schiff base) and its subsequent reduction with a borohydride reagent is particularly desirable, because the linkage has no stereoisomers and is chemically stable.

Reductive amination requires both amine and aldehyde reaction partners. Primary amino groups can be incorporated internally into short RNA oligonucleotides via solid-phase synthesis,[6,7] and oligonucleotides can be assembled into larger RNA molecules by ligation.[8−10] Here we have used 2′-amino-2′-deoxy-RNA (abbreviated 2′-NH_2-RNA) as the amine reaction partner. For generation of aldehydes on DNA, a few examples have been demonstrated at specific locations such as the 5′- or the 3′-termini,[11−19] an internal 2′-position,[20] a 1′-position of an abasic site,[21] and the nucleobases.[22] The termini of a DNA strand are appropriate modification sites because modifications at an internal 2′-position or at a nucleobase may unintentionally influence DNA duplex formation, which is the basis of the constraint approach.[1,2] Similarly, modifications are preferably made without alteration to the deoxyribose ring. We therefore focused on the termini to introduce DNA aldehydes.

Automated solid-phase DNA synthesis utilizes a variety of reaction conditions and reagents, some of which are incompatible with an unprotected aldehyde.[23] A 1,2-diol (glycol) is a stable precursor of an aldehyde, because the 1,2-diol can be cleaved oxidatively to the aldehyde using $NaIO_4$ after oligonucleotide synthesis. Utilizing this or other strategies, aldehydes have been generated both at the 5′- and 3′-ends of DNA in various ways (see structures **A**−**G**).[12−18] To minimize the exposure of the modified nucleoside to the solid-phase synthesis conditions and for ease of synthesis of oligonucleotides in the conventional 3′→5′ direction, installation of the aldehyde is preferred at the 5′- rather than the 3′-end (i.e., not **B** or **E**). In all of the published examples **A**−**G**, after reductive amination the tether that connects the DNA to the amine-bearing biomolecule was either relatively long and flexible (**A**−**D**) or short and rigid (**E**−**G**). A long tether would probably be a poor choice for connecting a structural constraint because flexibility could

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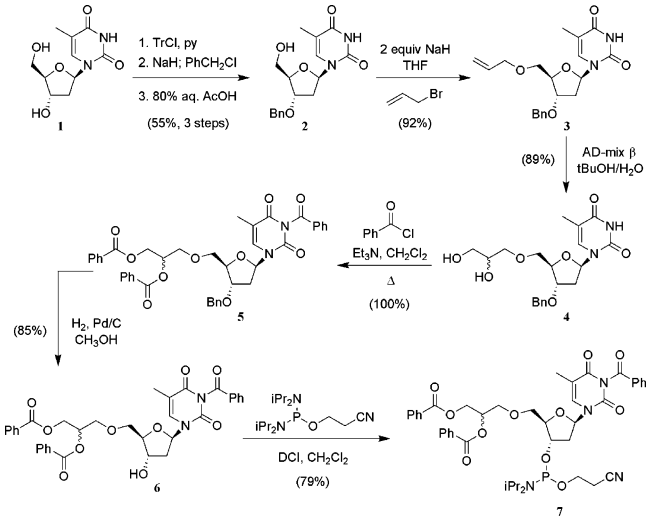
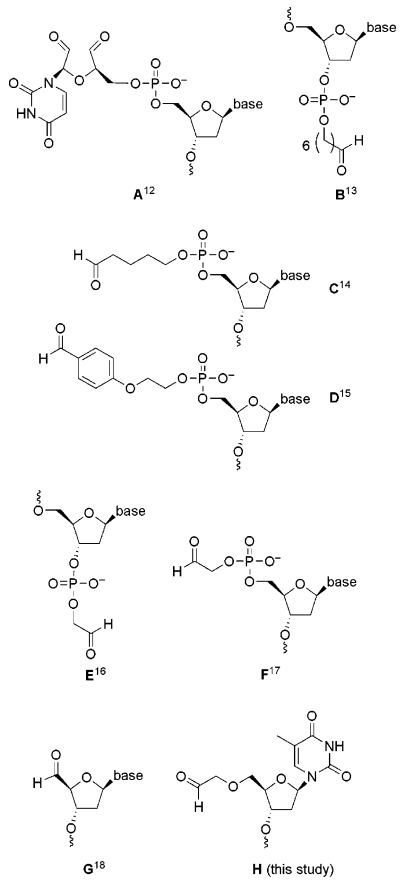
SCHEME 1. Synthesis of Phosphoramidite 7

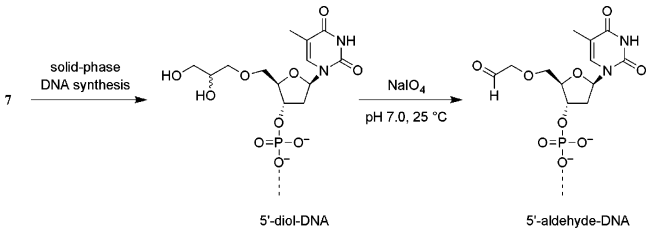


compromise the integrity of the constraint. In this report, an new short tether for connecting DNA and RNA (structure **H**) was designed and implemented. This was achieved via the synthesis of thymidine nucleoside phosphoramidite **7** that has its reactive aldehyde functionality masked as a 1,2-diol.[12−14,16−19]



The target phosphoramidite **7** was synthesized from thymidine in eight steps (Scheme 1). Thymidine **1** was converted to benzyl ether **2** in a three-step procedure starting with the protection of the 5′-OH as the triphenylmethyl ether followed by benzylation of the 3′-OH and subsequent deprotection of the triphenylmethyl

SCHEME 2. Synthesis of 5′-Aldehyde-DNA from Phosphoramidite 7 via 5′-Diol-DNA



ether.[24] Allylation of the 5′-OH of **2** with 2 equiv of NaH and 1 equiv of allyl bromide afforded compound **3** (2 equiv of NaH were necessary to deprotonate the 5′-OH because the uracil NH is more acidic). Asymmetric dihydroxylation (AD)[25] of the alkene **3** using AD-mix β afforded 1,2-diol **4** as an inseparable 2:1 mixture of diastereomers (absolute configurations were not determined). Although it was unnecessary to control the stereoselectivity of this dihydroxylation reaction because the new stereocenter would ultimately become the aldehyde carbon, AD-mix β was used for ease of reaction setup and purification. Diol **4** was treated with excess benzoyl chloride and triethylamine in refluxing methylene chloride to provide the trisbenzoylated nucleoside **5**. Although N^3-benzoyl protection is not required for use of the phosphoramidite in solid-phase synthesis, such protection is acceptable because the N^3-benzoyl group is readily removed by routine NH_4OH treatment. Catalytic hydrogenation of **5** to **6** followed by phosphitylation afforded the phosphoramidite **7** in an overall yield of 31% starting from thymidine.

Compound **7** was utilized as the final phosphoramidite during standard solid-phase DNA synthesis, with coupling yield >95% (using standard conditions except the coupling time was increased from 1.5 to 10 min). Treatment with NH_4OH to cleave the DNA from the solid support and to remove the benzoyl and nucleobase protecting groups afforded the desired DNA with a 5′-diol modification (5′-diol-DNA; Scheme 2).The 5′-diol-DNA was oxidized to 5′-aldehyde-DNA using $NaIO_4$. Following oxidative cleavage to the aldehyde, the excess $NaIO_4$ must be removed completely, because excess $NaIO_4$ would oxidize the 1,2-diol of the 3′-terminal ribose of the RNA during the reductive amination reaction. Sephadex G-25 size-exclusion columns were used for desalting the DNA following oxidation with $NaIO_4$. This approach was found to be effective in the recovery of 5′-aldehyde-DNA from the column while removing essentially all $NaIO_4$.

Reductive amination reactions are generally performed under acidic conditions to favor protonation of the intermediate imine, which is subsequently reduced to an amine using sodium cyanoborohydride.[26] These reactions are usually performed with excess amine and a limiting amount of the aldehyde. However, in the case of coupling modified DNA and RNA, a large excess of the 2′-NH_2-modified RNA is prohibitive, whereas excess DNA can more readily be used. In our initial experiments, typical reductive amination yields were only 15−20% even with a 1000× excess of 5′-aldehyde-DNA (data not shown). Reductive amination reactions involving organic molecules have been

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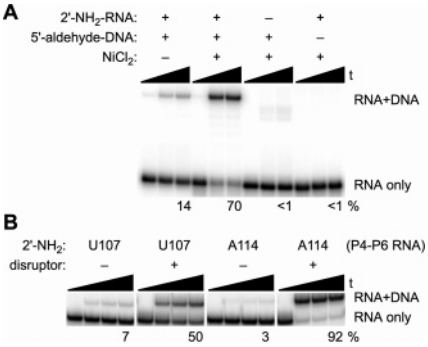

FIGURE 1. Reductive amination between 2′-NH_2-RNA and 5′-aldehyde-DNA: (A) Shown are reactions performed with 5′-^{32}P-labeled RNA and unradiolabeled 5′-aldehyde-DNA oligonucleotides, each of which were 15 nt in length (NaOAc pH 5.0, 10 mM $NaCNBH_3$, 2.5 mM $NiCl_2$ if present, 25 °C). In each set of lanes, the three timepoints were 0, 3, and 6 h (20% PAGE). (B) Shown are reactions performed with 5′-^{32}P-labeled P4−P6 RNA (160 nt) and unradiolabeled 15-nt 5′-aldehyde-DNA (NaOAc pH 5.0, 10 mM $NaCNBH_3$, 2.5 mM $NiCl_2$, 45 °C). In each set of lanes, the four timepoints were 0, 1.5, 3, and 6 h (8% PAGE). For 2′-NH_2 at P4−P6 nucleotide U107 and A114 without disruptor but with 25 mM $NiCl_2$, the yields were improved to 21% and 20%, although slight degradation was observed (data not shown).

improved by using Lewis acid catalysts to activate the aldehyde.[27] Therefore, we tested a number of divalent transition metal cations such as Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} (as their chloride and sulfate salts) in the reductive amination between the 5′-aldehyde-DNA and the 2′-NH_2-RNA. Because RNA is susceptible to degradation in the presence of high concentration of metal ions, the concentration of the metal ion and the temperature of the reaction were carefully optimized. The addition of 2.5 mM $NiCl_2$ (50× relative to DNA) at 25 °C was found to be optimal for increasing the reductive amination yield with minimum RNA degradation (Figure 1A, compare first two sets of lanes). No effect was observed when Ni^{2+} was replaced with monovalent metal ions such as Na^+ and K^+, even at 100 mM (data not shown). The mechanism of this Ni^{2+} enhancement of the reductive amination reaction was not pursued in detail.[28] Plausibly, Ni^{2+} may act as a Lewis acid to activate the 5′-aldehyde on the DNA for imine formation, which is thought to be rate-determining.[26] In addition, divalent metal ions such as Ni^{2+} could induce some tertiary structure in the RNA, which could indirectly enhance the rate of imine formation. If imine reduction is instead rate-determining, then Ni^{2+} must interact with the imine to increase its electrophilicity.

To corroborate that the reductive amination occurs between the 2′-NH_2-RNA and the 5′-aldehyde-DNA, control reactions were performed with substrates lacking one of the reactive functional groups. No reaction was observed when the RNA lacked the 2′-NH_2 or when the DNA lacked the 5′-aldehyde (Figure 1A, last two sets of lanes). Importantly, these experiments demonstrated that the exocyclic amine groups on the nucleobases do not react appreciably under our reductive amination conditions. The reductive amination reaction between 2′-NH_2-RNA and 5′-aldehyde-DNA could be performed on the preparative (5 nmol) scale, with ∼50% isolated yield after polyacrylamide gel electrophoresis (PAGE) purification. The product identity was confirmed by MALDI mass spectrometry (see Experimental Section).

Some reductive amination sites may be desirable within regions of RNA that have considerable secondary or tertiary structure. Acylation reactions of 2′-NH_2 groups in both RNA and DNA are dependent on local flexibility of the nucleic acid; for example, a decreased acylation rate was observed when the 2′-NH_2 group was present in a duplex secondary structure or involved in a tertiary contact.[29] To demonstrate that the reductive amination approach for DNA attachment works well for structured RNAs, we used the 160-nucleotide P4−P6 RNA domain of the *Tetrahymena* group I intron RNA as the 2′-NH_2-RNA partner (Figure 1B).[1,2] With the 2′-NH_2 at two different sites in P4−P6 (either U107 or A114), in both cases a preparatively useful yield of RNA−DNA product was obtained. The reductive amination yield was higher at 45 °C than at 25 °C, and increasing the Ni^{2+} concentration from 2.5 to 25 mM was modestly effective at improving the yield. Most important for a high yield of reductive amination product using the P4−P6 RNA was inclusion of a "disruptor" DNA oligonucleotide that is complementary to a substantial portion of the RNA sequence, thereby leaving the 2′-NH_2-RNA within a single-stranded region.

In conclusion, a new DNA phosphoramidite **7** was synthesized that permits the preparation of 5′-aldehyde-DNA in which the aldehyde carbon is connected via a single methylene group to the 5′-oxygen of the DNA (structure **H**). A convenient protocol for the oxidative cleavage of the 1,2-diol to the aldehyde was developed using $NaIO_4$, for which the excess was removed by size-exclusion chromatography. The covalent attachment of 5′-aldehyde-DNA to 2′-NH_2-RNA was achieved by reductive amination, resulting in a short 5-atom tether connecting the ribose rings of the DNA and the RNA. The optimized reaction conditions (including Ni^{2+} concentration, temperature, and inclusion of disruptor DNA oligonucleotide) were dependent on the 2′-NH_2-RNA reaction partner. The synthesis of the DNA phosphoramidite **7** and the development of the reductive amination protocol expand our ability to attach DNA (and perhaps small molecules with aldehyde functional groups) to biomolecules such as RNA.

Experimental Section

5′-*O*-Allyl-3′-*O*-benzylthymidine (3). A 60% dispersion of sodium hydride in mineral oil (0.68 g, 17.1 mmol) was added to a solution of **2**[24] (2.58 g, 7.7 mmol) in 20 mL of THF. The suspension was stirred at 60 °C for 2 h, and allyl bromide (0.67 mL, 7.7 mmol) was added at room temperature. The mixture was stirred at room temperature overnight and quenched with methanol (1 mL). The mixture was diluted with 50 mL of EtOAc, and the organic layer was washed with saturated $NaHCO_3$ (50 mL). The aqueous layer was extracted with EtOAc (2 × 25 mL). The combined organic extracts were washed with saturated aqueous NaCl (50 mL), dried over Na_2SO_4, and concentrated under vacuum. The resulting oil was purified via silica gel chromatography with 0−35% EtOAc in CH_2Cl_2 as the eluant to produce 2.64 g (92%) of **3** as a pale yellow syrup: R_f 0.35 (3:1 CH_2Cl_2/EtOAc); for ^{1}H and ^{13}C NMR, see Supporting Information. EI-HRMS: M^+ calcd for $C_{20}H_{24}N_2O_5$, 372.1685; found, 372.1688.

5′-*O*-(2,3-Dihydroxypropyl)-3′-*O*-benzylthymidine (4). AD-mix-β [20.1 g, containing 0.03 mmol $K_2OsO_2(OH)_4$] dissolved in

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(28) The reductive amination proceeds well with 2′-NH_2-C and A in addition to U. Reactions with 2′-NH_2-A are faster and require little or no Ni^{2+} for optimal reactivity (data not shown).

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70 mL of water and 70 mL of *t*-BuOH was stirred at room temperature to produce two clear phases. The reaction mixture was cooled to 0 °C, and a syrup of **3** (2.64 g, 7.12 mmol) dissolved in 20 mL of *t*-BuOH and 20 mL of water was added at once. The heterogeneous slurry was stirred vigorously at 4 °C overnight. The reaction was quenched by the addition of sodium sulfite (21 g). The mixture was allowed to warm to room temperature and was stirred for 30−60 min. The mixture was diluted with 75 mL of EtOAc and, after separation of the layers, the aqueous layer was washed with EtOAc (2 × 50 mL). The combined organic extracts were washed with saturated aqueous NaCl (75 mL), dried over Na_2SO_4, and concentrated under vacuum. The resulting paste was purified via silica gel chromatography with 0−5% MeOH in CH_2Cl_2 as the eluant to produce 2.56 g (89%, 2:1 ratio of unassigned diastereomers as estimated from ^{1}H NMR) of **4** as a white foam: R_f 0.6 (1:9 MeOH/CH_2Cl_2); for ^{1}H and ^{13}C NMR, see Supporting Information. EI-HRMS: M^+ calcd for $C_{20}H_{26}N_2O_7$, 406.1740; found, 406.1737.

5′-*O*-(2,3-Di-*O*-benzoylpropyl)-*N*^{3}-benzoyl-3′-*O*-benzylthymidine (5). A portion of **4** (0.20 g, 0.49 mmol) was coevaporated from pyridine (2 mL) and dissolved in CH_2Cl_2 (2 mL). Et_3N (0.70 mL, 5.07 mmol) and benzoyl chloride (0.57 mL, 5.01 mmol) were added, and the mixture was heated at reflux for 1 h. After cooling to room temperature, CH_2Cl_2 (20 mL) and saturated $NaHCO_3$ (20 mL) were added. After separation of the layers, the aqueous layer was washed with CH_2Cl_2 (2 × 15 mL). The combined organic extracts were washed with aqueous NaCl (30 mL), dried over Na_2SO_4, and concentrated under vacuum. The resulting paste was purified via silica gel chromatography with 0−10% EtOAc in CH_2Cl_2 to afford 0.36 g (100%) of **5** as a syrup: R_f 0.90 (1:9 EtOAc/CH_2Cl_2); for ^{1}H and ^{13}C NMR, see Supporting Information. EI-HRMS: M^+ calcd for $C_{41}H_{38}N_2O_{10}$, 718.2526; found, 718.2517.

5′-*O*-(2,3-Di-*O*-benzoylpropyl)-*N*^{3}-benzoylthymidine (6). A portion of 10% Pd−C (0.5 g, 0.45 mmol) was added to a solution of **5** (0.35 g, 0.48 mmol) in MeOH (4 mL). The flask was evacuated and placed in an atmosphere of hydrogen for 45 min. It was observed that the N^3-benzoyl group was partially removed under these conditions if the reaction time was prolonged (>90 min). The mixture was filtered, and the residue was purified via silica gel chromatography with 0−30% EtOAc in CH_2Cl_2 to afford 0.26 g (85%) of **6** as a pale yellow foam: R_f 0.15 (1:9 EtOAc/CH_2Cl_2); for ^{1}H and ^{13}C NMR, see Supporting Information. EI-HRMS: M^+ calcd for $C_{34}H_{32}N_2O_{10}$, 628.2056; found, 628.2063.

5′-*O*-(2,3-Di-*O*-benzoylpropyl)-*N*^{3}-benzoylthymidine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (7). A portion of **6** (0.15 g, 0.24 mmol) was coevaporated with pyridine (2 mL) and dissolved in CH_2Cl_2 (2.0 mL). 4,5-Dicyanoimidazole (DCI, 31 mg, 0.26 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (0.83 mL, 0.26 mmol) were added. The cloudy mixture was stirred for 4 h at room temperature and partitioned between CH_2Cl_2 (20 mL) and saturated aqueous $NaHCO_3$ (20 mL). After separation of the layers, the aqueous layer was extracted with CH_2Cl_2 (2 × 20 mL). The combined organic extracts were washed with aqueous NaCl (40 mL), dried over Na_2SO_4, and concentrated under vacuum. The resulting paste was purified via chromatography on Et_3N-washed silica gel with 1:2 to 1:1 CH_2Cl_2-hexanes containing 1% Et_3N to afford **7** (0.16 g, 79%) as a pale yellow foam: R_f 0.50 (1:1 CH_2Cl_2/hexanes containing 1% Et_3N); for ^{1}H and ^{13}C NMR, see Supporting Information. ^{31}P NMR (202 MHz, acetone-d_6) δ 149.68, 149.58, 149.24, 149.15. FAB-HRMS: $[M+H]^+$ calcd for $C_{43}H_{50}N_4O_{11}P$, 829.3213; found, 829.3210.

Preparation and Oxidation of 5′-Diol-DNA Oligonucleotides. Each DNA oligonucleotide modified with a 5′-diol was synthesized using phosphoramidite **7** and purified by 20% PAGE. During solid-phase synthesis, the standard coupling conditions were used except for an increase in the coupling time from 1.5 to 10 min; the coupling yield was >95% based on appearance of the abort band on PAGE. DNA sequences (5′-T modified as a diol): 5′-TTGACCATGATTCCG-3′ (Figure 1A); 5′-TTGACCCGCTCTCCT-3′ (Figure 1B). MALDI-MS (two sequences): calcd 4616.9, found 4612.3 and calcd 4528.9, found 4531.4. For oxidation to the 5′-aldehyde-DNA, to a solution of 5′-diol-DNA (6 nmol) in 200 mM sodium phosphate buffer, pH 7.0, was added $NaIO_4$ (1000× relative to DNA, added from an 0.2 M aqueous stock solution). The 50 μL sample was incubated at room temperature for 30 min, and excess $NaIO_4$ was removed with a Sephadex G-25 mini spin column (Amersham). The eluted material was evaporated to dryness, and the 5′-aldehyde-DNA (assumed 6 nmol) was used immediately in the reductive amination reaction. MALDI-MS for 5′-aldehyde-DNA (two sequences) calcd 4584.9, found 4587.6 and calcd 4496.9, found 4494.9.

Analytical-Scale Reductive Amination Reactions. 2′-NH_2-RNA (5 pmol, supplemented with a trace amount of 5′-^{32}P-labeled RNA) and 5′-aldehyde-DNA (500 pmol) were mixed in a total volume of 10 μL containing 100 mM NaOAc pH 5.0, 10 mM NaH_2PO_4 (as a general 5′-phosphatase inhibitor), 2.5 mM $NiCl_2$, and 10 mM $NaCNBH_3$ at 25 °C (the $NaCNBH_3$ was added last). For Figure 1A, the RNA sequence was 5′-GGAAUUGCGGGAAAG-3′ (the 2′-NH_2-uridine is underlined). For Figure 1B, the RNA sequence was that of the P4−P6 RNA[30] with a 2′-NH_2 at either U107 or A114, prepared by splint ligation using T4 DNA ligase as described.[1] When included, the disruptor DNA was complementary to P4−P6 nucleotides 175−225. Aliquots of 2 μL were withdrawn at appropriate timepoints and quenched onto a solution of 80% formamide, 1× TB [89 mM each Tris and boric acid (pH 8.3)], and 50 mM EDTA containing 0.25% each bromophenol blue and xylene cyanol. The quenched samples were electrophoresed on 20% or 8% PAGE and imaged on a phosphorimager.

Preparative-Scale Reductive Amination Reactions. 2′-NH_2-RNA (5 nmol; sequence from above) was mixed with 5′-aldehyde-DNA (10 nmol, combined from two preparations as above; sequence same as for Figure 1B as listed above) in a total volume of 167 μL containing 100 mM NaOAc (pH 5.0) 2.5 mM $NiCl_2$, and 10 mM $NaCNBH_3$ at 25 °C for 4 h (the $NaCNBH_3$ was added last). The colorless solution was quenched onto a 300 μL solution of 80% formamide, 1× TB, and 50 mM EDTA containing 0.25% each bromophenol blue and xylene cyanol, and the product was purified by 20% PAGE. A typical yield was 2.5 nmol of the reductive amination product. MALDI-MS for RNA-DNA product: calcd, 9399.0; found, 9394.1.

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Supporting Information Available: General experimental procedures; tabulation of ^{1}H and ^{13}C NMR spectral peaks for **3**−**7**; images of ^{1}H NMR spectra for **3**−**7**; and image of ^{31}P NMR spectrum for **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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